CHROM. 4817

Determination of steroids by densitometry of derivatives

I. Assay of estrogens as azobenzene-4-sulfonates

The assay of steroids in biological extracts, as carried out in most clinical routine laboratories, usually depends on more or less specific color reactions, the resulting chromogens being submitted to photometry or fluorometry for evaluation[1-3]. Increased specificity of such methods may be obtained by introduction of additional chromatographic procedures. On the other hand, the isolation of individual steroids prior to the color reaction requires their elution from the chromatogram, thus adding to the losses and limiting the capacity of the particular method. In order to overcome such drawbacks, the direct photometry of colored steroid derivatives has been proposed[4-6]; this not only allows easy quantitation but may also increase the sensitivity of the assay.

The present communication describes the estimation of estrogens after conversion into their azobenzene-4-sulfonic acid esters and densitometry of the derivatives on chromatoplates.

Methods

0.1 ml 0.1% azobenzene-4-sulfonyl chloride (ABS chloride) in dry acetone, 0.85 ml acetone and 0.05 ml 0.02 *N* sodium hydroxide are added to the dry residue containing 0.05–10.0 μg estrogen in a glass stoppered centrifuge tube. The reaction mixture is kept at 50–55° for 30 min, diluted with 20 ml ether and then extracted twice with 5 ml 0.1 *N* sodium hydroxide and twice with 5 ml water, before being filtered through anhydrous sodium sulfate and evaporated to dryness under nitrogen.

The residue is quantitatively transferred on to the chromatoplates (20 × 20 cm) coated with Silica Gel G (0.25 mm thickness; Merck AG, Darmstadt, G.F.R.) by means of chloroform or benzene. Ascending chromatography is performed in one of the solvent systems indicated in Table I, using 1 cm wide bands for the samples with empty bands in between. After drying the chromatograms thoroughly, the sample lanes are subjected to densitometry at 313 nm in a spectrodensitometer (SD 3000; Schoeffel Instruments Corp., Westwood, N.J., U.S.A.). The peaks registered can be evaluated on the basis of their height or area, as determined by triangulation, and by comparing them to corresponding values of standard material.

Results and discussion

When six 1.0 μg samples of 6,7-^{3}H-estrone (3-hydroxy-1,3,5-estratrien-17-one) or 6,7-^{3}H-estriol (1,3,5-estratriene-3,16α,17β-triol) with 6,270 c.p.m. and 12,770 c.p.m. ^{3}H, respectively, were subjected to the above reaction, the recovery of ^{3}H-activity from the final ether extracts amounted to 96.9 ± 2.8% for estrone and 94.2 ± 3.3% for estriol. After subsequent thin-layer chromatography of such extracts and elution of the derivatives, recoveries of between 85.4% and 87.1% of the original ^{3}H-activity were obtained, indicating significant losses during the additional steps. However, it may be concluded from these recovery experiments with labeled estrogens, that the reaction of the phenolic steroids with ABS chloride proceeded practically to completeness.

TABLE I

TLC R_F VALUES OF ESTROGENS AND THEIR ABS DERIVATIVES

Solvent systems: A = chloroform–benzene-ethanol (18 : 2 : 1); B = chloroform–dioxan (94 : 6); C = cyclohexane–ethyl acetate (3 : 1).

Solvent system	*Estrone*		*Estradiol*		*Estriol*	
	Free	*ABS*	*Free*	*ABS*	*Free*	*ABS*
A	0.48	0.67	0.34	0.51	0.05	0.14
B	0.55	0.62	0.29	0.35	0.02	0.08
C	0.44	0.57	0.17	0.28	0.02	0.07

The absorption spectra of the ABS derivatives of estrone, estradiol (1,3,5-estratriene-3,17β-diol) and estriol all exhibited absorption maxima at 323 nm and 446 nm, characteristic for the azobenzene group. The molar extinction coefficients ε at 323 nm were found to be between 28,300 and 28,400 for the three derivatives, whereas the ε values at 446 nm were only approximately 720. The almost identical ε values at 323 nm, corresponding to that of the reagent, obviously suggest that for all three derivatives tested one common hydroxy group, *viz.*, the phenolic 3-hydroxy group was esterified under the experimental conditions specified. This could be confirmed by the failure of ABS estradiol or ABS estriol to react with BARTON's reagent[7] or Fast Blue B salt. The R_F values of the free estrogens and their derivatives in different solvent systems are listed in Table I. Apparently, the replacement of the 3-hydroxy group by the azobenzene-4-sulfonate group does not cause a substantial change in polarity.

Multiple assays of 0.1–10.0 μg of estrone or estriol by densitometry of their ABS derivatives on chromatoplates yielded the results compiled in Table II. While the accuracy of the assay ranged between 92 and 95%—comparable to the data of the recovery experiments with labeled estrogens—its precision did not exceed 6.1%

TABLE II

THE RELATIONSHIP BETWEEN PEAK AREA AND CONCENTRATION OF ESTRONE OR ESTRIOL AS THEIR ABS DERIVATIVES

s = sensitivity setting of the instrument; n = number of determinations.

Amount of estrogen (μg)	s	*Peak area in cm² and standard deviation from the mean*			
		n	*ABS estrone*	n	*ABS estriol*
0.10	0.2	5	0.99 ± 0.06	5	0.93 ± 0.05
0.25		5	2.34 ± 0.14	5	2.40 ± 0.14
0.50		5	4.55 ± 0.26	5	4.53 ± 0.27
0.75		5	6.80 ± 0.36	5	7.02 ± 0.38
1.00		4	9.12 ± 0.47	4	9.04 ± 0.49
1.0	1.0	6	1.24 ± 0.07	5	1.21 ± 0.08
2.5		5	2.94 ± 0.15	5	2.90 ± 0.14
5.0		5	5.72 ± 0.26	5	5.45 ± 0.23
7.5		5	8.30 ± 0.37		
10.0		5	10.15 ± 0.43		

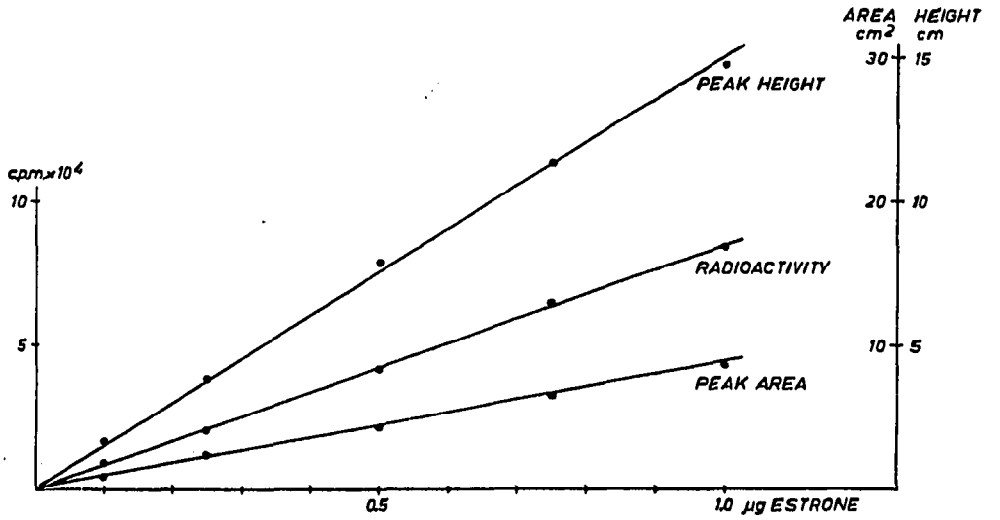

Fig. 1. Calibration curve for the estimation of estrones as their ABS derivatives, using 6,7-^{3}H-estrone. Densitometry at 313 mμ; slit width 0.2 mm; sensitivity 0.2, paper speed 4 in./min).

at the lower levels and 4.7% at the higher concentrations. A typical calibration curve set up for the estimation of estrone, is shown in Fig. 1. Although the absorption maximum of the ABS derivatives lies near 323 nm, densitometry at 313 nm led to slightly higher values than the same measurements at 323 nm, due to the intensity of the mercury line of the light source at 313 nm. The sensitivity of the method was estimated as approaching 50 ng from the ε values of the derivatives and the height or the area of the peak per μg of estrogen. By performing the densitometry at the highest sensitivity of the instrument (sensitivity setting: 0.1 instead of 0.2) the peak height or area per unit weight of estrogen can be almost doubled. However, unless uniform layers and dividing lines between the bands are obtainable, the background at such a sensitivity is apt to interfere with reproducible measurements. Likewise, the evaluation of the peaks by their height depends as much on adequate chromatographic techniques as on the speed of the recorder.

In view of the experimental data obtained, the present assay appears to be suitable for the analysis of estrogens like estrone, estradiol or estriol in pregnancy urine as will be shown in a forthcoming publication.

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